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(1390 REV. 5-93) US DEPT. OF COMMERCE PATENT & TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER
105454

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (if known, sec 37 C.F.R.1.5)
09/486037

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR98/01717	July 31, 1998	August 20, 1997

TITLE OF INVENTION
CULTURE AND IDENTIFICATION MEDIA SPECIFIC OF DIFFERENT SPECIES OF CANDIDA AND ANALYSIS METHODS

APPLICANT(S) FOR DO/EO/US
Sylvain ORENGA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A FIRST preliminary amendment.

    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A small entity statement.

16. ☐ Other items or information:

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U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) 09/486037	INTERNATIONAL APPLICATION NO. PCT/FR98/01717	ATTORNEY'S DOCKET NUMBER 105454

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO.....$840.00 International preliminary examination fee paid to USPTO (37 CFR1.482).....$670.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....$690.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$970.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$ 96.00	$840.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$840.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	24- 20 =	4	X $ 18.00	$72.00	
Independent Claims	2- 3 =	0	X $ 78.00	$	
Multiple dependent claim(s)(if applicable)			+ $260.00	$	
TOTAL OF ABOVE CALCULATIONS =				$912.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28). -				$	
SUBTOTAL =				$912.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 month from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$912.00	
				Amount to be refunded	$
				Charged	$

a. ☒ Check No. 106372 in the amount of $912.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 15-0461. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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(1390 Rev.8-93)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Sylvain ORENGA

U.S. National Stage of PCT/FR98/01717

Filed: February 18, 2000 Docket No.: 105454

For: CULTURE AND IDENTIFICATION MEDIA SPECIFIC OF DIFFERENT SPECIES OF CANDIDA AND ANALYSIS METHODS

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
Washington, D. C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

IN THE CLAIMS:

Please amend the claims as follows:

1. (Amended) [Medium for the culturing and specific identification of yeasts,] Culture medium for the specific identification and/or differentiation of *Candida albicans* and *Candida tropicalis* yeast, comprising a chromogenic or flourigenic substrate which can be hydrolyzed by an enzyme of the hexosaminidase family, **characterized in that** the medium also comprises at least one compound which selectively inhibts the hexosaminidase activity of *c tropicalis*.

Claim 4, line 1, change "Claims 1 to 3" to --Claim 1--.

Claim 5, line 1, change "Claims 1 to 4" to --Claim 1--.

Claim 6, line 1, change "Claims 1 to 5" to --Claim 1--.

Claim 8, line 1, change "any of the preceding claims" to --Claim 1--.

Claim 10, line 1, change "Claims 1 to 9" to --Claim 1--.

Claim 11, line 1, change "Claims 9 and 10" to --Claim 9--.

Claim 12, line 1, change "Claims 10 and 12" to --Claim 10--.

13. (Amended) [Medium for the detection and specific identification of yeasts,] Medium for the specific identification and/or differentiation of *candida albicans* and *Candida tropicalis* yeasts, characterized in that it comprises two substrates, a first chromogenic or fluorigenic substrate which can be hydrolyzed by an enzyme from the hexosaminidase family, and a second chromogenic or fluorigenic substrate which can by hydrolyzed by an enzyme from the glucosidase family.

Claim 15, line 1, change "either of Claims 13 and 14" to --Claim 13--.

Claim 16, line 4, delete "of any one of Claims 1 to 12".

Claim 17, line 1, change "any one of Claims 13 to 16" to --Claim 13--.

Claim 18, line 1, change "any one of Claims 1 to 17" to --Claim 1--.

Claim 19, line 7, change "any on of Claims 1 to 12" to --Claim 1--.

Claim 20, line 5, change "either of Claims 13 and 18" to --Claim 13--.

Claim 21, line 5, delete "according to either of Claims 15 and 16".

Claim 22, line 5, delete "according to either of Claims 15 and 16".

Claim 23, lines 1-2, change "any one of Claims 20 to 22" to --Claim 20--.

Claim 24, lines 1-2, change "any one of Claims 20 to 23" to --Claim 20--.

REMARKS

Claims 1-24 are pending. By this Preliminary Amendment, claims 1 and 13 are amended and 4-6, 8, 10-13 and 15-24 are amended to eliminate multiple dependencies. Prompt and favorable examination on the merits is respectfully solicited.

Respectfully submitted,

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MEDIA FOR THE CULTURING AND SPECIFIC IDENTIFICATION OF VARIOUS CANDIDA SPECIES, AND ANALYTICAL PROCESSES

The present invention relates to a medium for the culturing and specific identification of yeasts and to a microbiological analysis process to specifically identify *Candida albicans* and *Candida tropicalis* yeasts and/or to differentiate *C. albicans* and *C. tropicalis* yeasts.

The *C. albicans* species is the one most commonly isolated from clinical samples and gives rise to more or less extensive infections of the skin, the nails and mucous membranes in individuals with normal immune defenses and very serious infections in weakened individuals, and in particular those infected with the Human Immunodeficiency Virus (HIV). According to studies, *C. tropicalis* is the second or third most common species isolated in samples of human origin. It is thus essential not only to be able very rapidly to detect the presence of these yeasts in samples, but also to differentiate those belonging to the *C. albicans* species and those belonging to the *C. tropicalis* species.

To do this, numerous techniques have been proposed in recent years for rapidly identifying *C. albicans* yeasts. Most of these techniques are based on the demonstration of hexosaminidase activity, i.e. of enzymes with N-acetyl-β-D-glucosaminidase or N-acetyl-β-D-galactosaminidase or N-acetyl-β-D-mannosaminidase activity (FR-2 684 110, FR-2 659 982). However, these processes suffer from reduced specificity with respect to yeasts of the *C. tropicalis* species.

The inventors of the present invention have discovered that by inhibiting an enzymatic activity of the *C. tropicalis* species, in particular the hexosaminidase activity, it is possible to overcome the drawbacks of the abovementioned tests and thus to provide a quick and inexpensive means for identifying

and/or differentiating yeasts, in particular *C. albicans* and *C. tropicalis*.

Moreover, the glucosidase enzymatic activity has already been the subject of research in certain documents, such as Casal, M. and Linares, M.J. "Contribution to the study of the enzymatic profiles of yeast organisms with medical interest" Mycopathology 81, 155-159 (1983). This activity is positive in a number of strains of *C. albicans*, *C. tropicalis* and *Candida pseudotropicalis* (nowadays known as *Candida kefyr*), but negative for other *Candida* species, for example *C. parapsilosis*, *C. guilliermondii* and *C. krusei*.

It thus appeared to be advantageous to attempt to cumulate, in the same medium, the possibility of investigating two different enzymatic activities, i.e. hexosaminidase and glucosidase activities. Now, it is found that, in the media according to the invention, this cumulation makes it possible to differentiate more specifically *C. albicans* from *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and/or *C. tropicalis* and from other *Candida* species, but also to differentiate *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and/or *C. tropicalis* from other *Candida* species.

Needless to say, it is envisaged to combine, in the same medium, an inhibitor according to the invention, and even an activator of hexosaminidase activity, with the substrates specific for the hexosaminidase and glucosidase activities.

The subject of the invention is thus a medium for the culturing and the specific identification of yeasts, comprising a chromogenic or fluorigenic substrate which can be hydrolyzed by an enzyme of the hexosaminidase family, characterized in that the medium also comprises at least one compound which selectively inhibits the hexosaminidase activity of *Candida tropicalis*.

By virtue of the invention, the culture medium especially allows the specific identification of yeasts of the *C. albicans* and/or *C. tropicalis* species.

According to one preferred embodiment of the invention, the culture medium comprises, as selective inhibitor compound, an amide of formula (I):

(I) $R-(CO-NR'R'')_n$

in which, firstly, either R, R' and R'', independently of each other, consist of:

- a hydrogen atom,

- a saturated or unsaturated, aliphatic or cyclic hydrocarbon-based chain optionally comprising at least one hetero atom,

or each of the radicals R and/or R' and/or R'' together form a cyclic, saturated or unsaturated hydrocarbon-based chain optionally comprising at least one hetero atom,

and, secondly, n is an integer greater than or equal to 1.

According to the invention, the expression "hydrocarbon-based chain *"comprising"* at least one hetero atom" means that the hydrocarbon-based chain can be substituted with at least one substituent such as, in particular, $-NH_2$, -COOH, -SH and a halogen atom, and/or can be interrupted with at least one hetero atom such as, in particular, O, S and N.

According to one preferred embodiment of the invention, the culture medium comprises, as selective inhibitor compound, an amide of formula (I):

(I) $R-(CO-NR'R'')_n$

in which, firstly, either R, R' and R'', independently of each other, consist of:

- a hydrogen atom,

- a saturated or unsaturated, aliphatic or cyclic hydrocarbon-based chain optionally interrupted by at least one hetero atom,

or each of the radicals R and/or R' and/or R'' together form a cyclic, saturated or unsaturated

hydrocarbon-based chain optionally comprising at least one hetero atom,

and, secondly, n is an integer greater than or equal to 1.

According to another preferred embodiment of the invention, the culture medium comprises, as selective inhibitor compound, an amide of formula (I):

(I) $R-(CO-NR'R'')_n$

in which, firstly, R, R' and R'', independently of each other, consist of:

- a hydrogen atom,
- an aliphatic hydrocarbon-based chain,

and, secondly, n is equal to 1 or 2.

According to a very preferred embodiment of the invention the selective inhibitor compound is an acetamide.

According to another embodiment of the invention, the culture medium comprises an activator which is specific for the hexosaminidase enzyme of *C. albicans*.

According to one preferred embodiment of the invention, the activator which is specific for the hexosaminidase enzyme is N-acetylglucosamine.

According to another embodiment of the invention, the culture medium comprises a mixture of selective inhibitor compounds.

According to one preferred embodiment of the invention, the mixture of selective inhibitor compounds consists of acetamide and formamide.

According to one preferred embodiment of the invention, the medium is liquid or gelled.

According to one embodiment of the invention, the culture medium is gelled and comprises, per liter:

- peptones or a mixture of peptones	0.01-40 g
- yeast extract	0.01-40 g
- glucose (source of carbon)	0-10 g
- phosphate buffer (pH between 5 and 8.5)	2.5-100 mM

- 5-bromo-4-chloro-3-indolyl-N-acetyl-β-D-glucosaminide $20\text{-}600 \times 10^{-6}$ M
- acetamide 0.01-20 g
- bacterial inhibitor 0-20 g
- agar 11-20 g

According to another preferred embodiment of the invention, the gelled or liquid culture medium described above furthermore comprises N-acetyl-glucosamine at a concentration of 1.0 g/l.

According to another preferred embodiment of the invention, the gelled or liquid culture medium described above furthermore comprises formamide at a concentration of 0.5 g/l.

Another subject of the invention is a microbiological analysis process for selectively identifying *C. albicans* and/or *C. tropicalis* yeasts and/or for differentiating *C. albicans* and *C. tropicalis* yeasts, characterized in that the sample to be analyzed is placed directly in contact with at least one identification medium described above.

To this end, the present invention also relates to a medium for detecting and specifically identifying yeasts, which is characterized in that it comprises two substrates, a first chromogenic or fluorigenic substrate which can be hydrolyzed by an enzyme from the hexosaminidase family, and a second chromogenic or fluorigenic substrate which can be hydrolyzed by an enzyme from the glucosidase family.

According to one preferred embodiment of the invention, in this medium, each substrate consists of a specific portion of the enzyme and of a marker portion, characterized in that the marker portion of the first substrate is different from the marker portion of the second substrate.

According to another preferred embodiment of the invention, the medium comprises a hexosaminidase activator and/or inhibitor.

When there is an activator and/or an inhibitor, this activator consists of a hexosamine and/or a

hexosaminidine and this inhibitor takes the characteristics described above.

According to yet another preferred embodiment of the invention, the hexosaminidinase substrate consists of an indoxyl derivative and/or the glucosidase substrate consists of an indoxyl derivative.

In all cases, the medium is liquid or gelled.

The present invention also relates to a microbiological analysis process for detecting and selectively identifying certain species of *Candida* yeasts, which is characterized in that the sample is placed in direct contact with a medium according to either of Claims 13 and 18, time is allowed for colorations to appear in the medium, and identification is made, on the basis of the differences in coloration, of the *C. albicans* species from, on the one hand, the *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and/or *C. tropicalis* species, and, on the other hand, from the other *Candida* species, and of the *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and/or *C. tropicalis* species from the other *Candida* species.

When the medium contains no activator or inhibitor, a waiting period of between 36 and 60 hours and advantageously essentially 48 hours is allowed.

When the medium contains an activator or an inhibitor, a waiting period of between 18 and 30 hours and advantageously essentially 24 hours is allowed.

According to a first embodiment, these processes make it possible to identify *C. albicans*, *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and/or *C. tropicalis* from other *Candida* species, when the medium contains:

- a hexosaminidase substrate, and/or
- a glucosidase substrate, and/or
- a hexosaminidase activator, and/or
- a hexosaminidase inhibitor.

According to a second embodiment, these processes make it possible to identify *C. albicans* from

C. guilliermondii, *C. kefyr*, *C. lusitaniae*, *C. tropicalis* and/or other *Candida* species, when the medium contains:

- a hexosaminidase substrate and a glucosidase substrate, and/or
- a hexosaminidase activator, and/or
- a hexosaminidase inhibitor.

The expression "compound which selectively inhibits the hexosaminidase activity of *C. tropicalis*" means any compound capable of selectively inhibiting the hexosaminidase activity of *C. tropicalis*. For example, the compounds of amide type of the formula described above have the property of specifically inhibiting the hexosaminidase activity of *C. tropicalis* without affecting that of *C. albicans*.

The term "identification" means detection and/or quantification.

The term "sample" in particular means any sample of biological type taken, a yeast strain or a set of yeast strains isolated, for example, after culturing.

The composition of the culture medium, expressed in g/l of final medium, is outlined below in general terms.

The medium comprises a nutrient base required for the growth of yeasts and inhibitors specific for the hexosaminidase of *C. tropicalis* according to the invention.

The constituent elements of the nutrient base comprise:

- from 0.01 to 40 g/l of peptones, such as meat peptone, the product sold by the company bioMérieux under the brand name bioSoyase or the like, or alternatively a mixture of peptones; preferably, the peptone or the mixture of peptones is present in the medium at a concentration of about 6 g/l ± 0.5 g/l;
- from 0.01 to 40 g/l, preferably about 1.5 g/l, of a yeast extract, supplying vitamins for the growth of the yeasts;

- a source of carbon, such as glucose, glycerol, an acetate, a pyruvate, a lactate, arginine, an aminobutyrate or a mixture of these components, in a proportion of from 0 to 10 g/l; the carbon source is preferably glucose in an amount of 1 g/l;

- a buffer added to the medium to give a pH which is favorable for the growth of *C. albicans*, of between 5 and 8.5; the buffer is chosen from phosphate buffer, Tris buffer, Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer and citrate buffer, in a proportion of from 2.5 to 100 mM; preferably, the buffer is a 10 mM phosphate buffer to adjust the pH of the medium to a value in the region of 7;

- from 11 to 20 g/l, preferably 15 g/l, of agar.

The chromogenic or fluorigenic substrate can be any chromogenic or fluorigenic substrate which can be hydrolyzed with a hexosaminidase, such as a galactosaminidase, glucosaminidase or mannosaminidase, to release a colored or fluorescent product. Preferably, the substrate is chosen from those showing strong coloration or fluorescence with few molecules, and inducing no change in the metabolism of the microorganisms, except for the desired enzymatic activity. These substrates are preferably chosen, for the chromogenic substrates, from those comprising a chromophoric group such as a substituted or unsubstituted indolyl, and in particular from 5-bromo-4-chloro-3-indolyl-N-acetyl-β-D-glucosaminide, 5-bromo-4-chloro-3-indolyl-N-acetyl-β-D-galactosaminide, 6-chloro-3-indolyl-N-acetyl-β-D-glucosaminide and 5-bromo-6-chloro-3-indolyl-N-acetyl-β-D-glucosaminide of from 20 to 600 mM, advantageously 200 mM 5-bromo-4-chloro-3-indolyl-N-acetyl-β-D-glucosaminide, and for the fluorigenic substrates, from 4-methylumbelliferyl-N-acetyl-b-D-galactosaminide and 4-methylumbelliferyl-N-acetyl-b-D-glucosaminide.

The inhibitor which is specific for the hexosaminidase of yeasts of the *C. tropicalis* species

is preferably chosen from the group of compounds of amide type (I) or mixtures thereof. It is chosen in particular from amides such as formamide, acetamide, propionamide, glycinamide, succinamide and the like. The amount of compound of amide type is between 0.01 and 20 g/l. Preferably, the inhibitor chosen is 1 g/l acetamide.

In order to obtain an intense and early activity for the yeasts of the *C. albicans* species, a hexosaminidase activator can advantageously be added to the culture medium, as described in document FR-A-2 684 110. Similarly, a bacterial inhibitor or a mixture of bacterial inhibitors, for inhibiting the growth of Gram-positive bacteria and that of Gram-negative bacteria, without affecting the growth of the yeasts, and if possible of fungi, can be added to the medium. Preferably, the bacterial inhibitors are chosen from the group of antibiotics such as gentamycin, chloramphenicol, penicillin, streptomycin, cycloheximide, neomycin, tetracycline, oxytetracycline or a mixture of antibiotics, and/or from tellurite, a molybdate and the like, or mixtures thereof. Advantageously, chloramphenicol (0.5 g/l) or a mixture of gentamycin (0.1 g/l) and chloramphenicol (0.05 g/l) is chosen. It is also possible to inhibit the growth of the bacteria by reducing the pH of the medium to an acidic pH.

As is demonstrated in the examples below, the enzymatic hydrolysis reaction remains specific beyond the 24 hours of incubation.

Example 1:

Tests were carried out to examine the effect of acetamide on the hexosaminidase activity of yeasts.

Two media were prepared according to the usual techniques. The first medium below, referred to as Medium I, contains all the elements of the nutrient base, as well as a chromogenic substrate for a hexosaminidase and a bacterial inhibitor mixture.

The composition of Medium I, per liter of final medium, is as follows:

- bioSoyase (bioMérieux)..... 6.0 g
- yeast extract (bioMérieux)..... 1.5 g
- glucose (Merck)..... 1.0 g
- phosphate buffer (Merck)..... 10.0 mM
- Mn2+ (Merck)..... 1.0 mM
- 5-bromo-4-chloro-3-indolyl-N-acetyl-β-D-glucosaminide (Biosynth)..... 0.1 g
- gentamycin..... 0.1 g
- chloramphenicol..... 0.05 g
- agar (bioMérieux)..... 15.0 g

The pH of the medium was adjusted to about 7.

The second medium, referred to as Medium II, corresponds to the medium according to the invention and contains all the elements described above for Medium I, plus the inhibitor which is specific for the hexosaminidase of *C. tropicalis*, i.e. an acetamide compound (Sigma) at 1.0 g.

12 strains of yeast were cultured directly in a Petri dish on these two media. The strains from the Applicant's collection belong to the following species: *C. albicans* (3 strains), *C. glabrata* (2 strains), *C. krusei* (1 strain), *C. parapsilosis* (1 strain), *C. tropicalis* (3 strains), *Saccharomyces cerevisiae* (1 strain), *Trichosporon spp.* (1 strain). The dishes were incubated at 37°C for 48 hours. The colonies formed were examined visually, after incubation for 24 and 48 hours, respectively, according to the following interpretations:

- the blue colonies correspond to strains producing N-acetyl-β-D-glucosaminidase, belonging in principle to the species *C. albicans*;
- the white colonies correspond to strains not producing the abovementioned enzyme or strains in which this enzyme is inhibited by the compound of amide type, these colonies thus belonging to other yeast strains,

which will in this case be identified using the usual techniques.

The results are given in Table I below:

TABLE I

Species	Medium	Coloration at 24 hours			Coloration at 48 hours		
		Strong	Weak	None	Strong	Weak	None
C. albicans	I	1*	2	-	3	-	-
	II	1	2	-	3	-	-
C. glabrata	I	-	-	2	-	-	2
	II	-	-	2	-	-	2
C. krusei	I	-	-	1	-	-	1
	II	-	-	1	-	-	1
C. parapsilosis	I	-	-	1	-	-	1
	II	-	-	1	-	-	1
C. tropicalis	I	-	-	3	3	-	-
	II	-	-	3	-	1	2
S. cerevisiae	I	-	-	1	-	-	1
	II	-	-	1	-	-	1
Trichosporon	I	-	-	1	1	-	-
	II	-	-	1	1	-	-

* : number of strains, "-" = 0

As emerges from Table I above, supplying the compound of amide type allows a specific detection of the *C. albicans* strains, since only the *C. albicans* strains, as well as one strain of *Trichosporon* after incubation for only 48 hours, produce colored colonies on the medium according to the invention. The *C. tropicalis* colonies which are blue after 48 hours on Medium I give colorless colonies on Medium II, apart from a very faint coloration after incubation for 48 hours.

Example 2:

The experiment of Example 1 was repeated, but using liquid media instead of gelled media. Media III and IV thus correspond to Media I and II of Example 1, but contain no agar. Moreover, the concentration of 5-bromo-4-chloro-3-indolyl-N-acetyl-b-D-glucosaminide is 150 mg/l of final medium for a use in liquid medium. The media were distributed into glass ampules, at a rate of 3 ml per ampule. The strains studied are the same as in Example 1. A suspension calibrated to 2 on the MacFarland scale using a nephelometer was prepared for each of the strains directly in the ampules containing the media. The ampules thus inoculated were incubated for 48 hours at 37°C. They were examined after 24 and 48 hours, respectively, according to the interpretations of Example 1.

The results are given in Table II below:

TABLE II

Species	Medium	Coloration at 24 hours			Coloration at 48 hours		
		Strong	Weak	None	Strong	Weak	None
C. albicans	III	1*	2	-	3	-	-
	IV	1	2	-	3	-	-
C. glabrata	III	-	-	2	-	-	2
	IV	-	-	2	-	-	2
C. krusei	III	-	-	1	-	-	1
	IV	-	-	1	-	-	1
C. parapsilosis	III	-	-	1	-	-	1
	IV	-	-	1	-	-	1
C. tropicalis	III	-	2	1	3	-	-
	IV	-	-	3	-	2	1
S. cerevisiae	III	-	-	1	-	-	1
	IV	-	-	1	-	-	1
Trichosporon	III	-	-	1	-	1	-
	IV	-	-	1	-	1	-

* : number of strains, "-" = 0

As emerges from Table II above, supplying the amide compound allows a specific detection of the *C. albicans* strains. Specifically, after incubation for 24 hours, only the *C. albicans* strains give tubes colored blue in the medium according to the invention. The *C. tropicalis* strains, which give colored tubes in Medium III, give colorless tubes in Medium IV. After incubation for 48 hours, the coloration of the tubes containing *C. tropicalis* strains is also inhibited or at least very greatly reduced.

Example 3:

Tests were carried out to examine the effect of acetamide on the hexosaminidase activity of yeasts in the presence of an activator which is specific for this enzyme.

The experiment of Example 1 was reproduced, but with N-acetylglucosamine added to the medium. Media V and VI thus correspond to Media I and II of Example 1, to which N-acetylglucosamine has been added to a concentration of 1.0 g/l of final medium. The strains studied are the same as in Example 1. They were cultured directly in Petri dishes. The dishes were incubated at 37°C for 48 hours. The colonies formed were examined visually, after incubation for 24 and 48 hours, respectively, according to the interpretations of Example 1.

The results are given in Table III below:

TABLE III

		Coloration					
		at 24 hours			at 48 hours		
Species	Medium	Strong	Weak	None	Strong	Weak	None
C. albicans	V	2*	1	-	3	-	-
	VI	2	1	-	3	-	-
C. glabrata	V	-	-	2	-	-	2
	VI	-	-	2	-	-	2
C. krusei	V	-	-	1	-	-	1
	VI	-	-	1	-	-	1
C. parapsilosis	V	-	-	1	-	-	1
	VI	-	-	1	-	-	1
C. tropicalis	V	-	-	3	3	-	-
	VI	-	-	3	-	-	3
S. cerevisiae	V	-	-	1	-	-	1
	VI	-	-	1	-	-	1
Trichosporon	V	-	-	1	1	-	-
	VI	-	-	1	1	-	-

* : number of strains, "-" = 0

As emerges from Table III above, supplying the compound of amide type allows a specific detection of the *C. albicans* strains. Specifically, only the *C. albicans* strains, as well as one strain of *Trichosporon* after incubation for 48 hours only, produce colored colonies on the medium according to the invention. The *C. tropicalis* strains which are blue on Medium V give colorless colonies on Medium VI. These two media together thus also allow a specific identification of yeasts of the *C. tropicalis* species, since, after incubation for 48 hours, they are the only ones which are positive on Medium V and negative on Medium VI.

Example 4:

Tests were carried out to examine the effect of a mixture of amide compounds on the hexosaminidase activity of yeasts in the presence of an activator which is specific for this enzyme.

The experiments of Example 3 was reproduced, but with formamide at a concentration of 0.5 g/l of final medium (Medium VIII) being added to Medium VII, Medium VII being identical to Medium V of Example 3. The strains studied are the same as those in Example 3. They were cultured directly in Petri dishes. The dishes were incubated at 37°C for 48 hours. The colonies formed were examined visually, after incubation for 24 and 48 hours, respectively, according to the interpretations of Example 1.

The results are given in Table IV below:

TABLE IV

Species	Medium	Coloration at 24 hours			Coloration at 48 hours		
		Strong	Weak	None	Strong	Weak	None
C. albicans	VII	2*	1	-	3	-	-
	VIII	2	1	-	3	-	-
C. glabrata	VII	-	-	2	-	-	2
	VIII	-	-	2	-	-	2
C. krusei	VII	-	-	1	-	-	1
	VIII	-	-	1	-	-	1
C. parapsilosis	VII	-	-	1	-	-	1
	VIII	-	-	1	-	-	1
C. tropicalis	VII	-	-	3	3	-	-
	VIII	-	-	3	-	-	3
S. cerevisiae	VII	-	-	1	-	-	1
	VIII	-	-	1	-	-	1
Trichosporon	VII	-	-	1	1	-	-
	VIII	-	-	1	-	1	-

* : number of strains, "-" = 0

As emerges from Table IV above, supplying a second compound of amide type allows an even more specific detection of the *C. albicans* strains, since only the *C. albicans* strains produce colonies that are significantly colored on the medium according to the invention. The *C. tropicalis* strains which are blue on Medium VII give colorless colonies on Medium VIII, and the *Trichosporon* strain which is highly colored after incubation for 48 hours on Medium VII is only very faintly colored on Medium VIII.

Example 5:

Tests were carried out to examine the advantage of combining a hexosaminidase substrate and a β-glucosidase substrate in media for isolating and identifying yeasts.

A β-glucosidase substrate, 6-chloro-3-indolyl-β-D-glucoside, was added at a concentration of 0.07 g/l to Medium I of Example 1 (Medium IX). To this medium was added either a hexosaminidase activator (N-acetyl-glucosamine) at 1 g/l (Medium X), or an inhibitor of the hexosaminidase of *C. tropicalis* (acetamide) at 1 g/l (Medium XI), or a combination of the abovementioned activator and inhibitor at the same concentrations (Medium XII).

Eighteen strains of yeast were cultured directly in Petri dishes on these four media. The strains from the Applicant's collection belong to the following species: *C. albicans* (3 strains), *C. glabrata* (2 strains), *C. guilliermondii* (2 strains), *C. kefyr (2 strains)*, *C.* krusei (1 strain), C. *lusitaniae* (2 strains), *C. parapsilosis* (1 strain), *C. tropicalis* (3 strains), *Saccharomyces cerevisiae* (1 strain), *Trichosporon spp.* (1 strain). The dishes were incubated at 37°C for 48 hours. The colonies formed were examined visually, after incubation for 24 and 48 hours, respectively, according to the following interpretations:

- the blue colonies correspond to strains producing N-acetyl-β-D-glucosaminidase, belonging in principle to the *C. albicans* species;

- the pink colonies correspond to strains producing β-D-glucosidase, belonging in principle to the *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and *C. tropicalis* species;

- the mauve colonies correspond to strains producing the two enzymatic activities;

- the white colonies correspond to strains producing none of the abovementioned enzymes or to strains in which these enzymes are inhibited, and they thus belong to other yeast species which will in this case be identified using the usual techniques.

The results are given in Table V below:

TABLE V

Species	Medium	Coloration at 24 hours: Strong	Weak	None	Coloration at 48 hours: Strong	Weak	None
C. albicans	IX	1-blue	2-blue	-	3-blue	-	-
	X	2-blue	1-blue	-	3-blue	-	-
	XI	1-blue	2-blue	-	3-blue	-	-
	XII	2-blue	1-blue	-	3-blue	-	-
C. glabrata	IX	-	-	2	-	-	2
	X	-	-	2	-	-	2
	XI	-	-	2	-	-	2
	XII	-	-	2	-	-	2
C. guilliermondii	IX	-	-	2	2-pink	-	
	X	-	-	2	2-pink	-	2
	XI	-	-	2	2-pink	-	2
	XII	-	-	2	-	2-pink	2
C. kefyr	IX	-	2-pink	2	2-pink	-	2
	X	-	2-pink	2	2-pink	-	2
	XI	-	2-pink	2	2-pink	-	2
	XII	-	2-pink	2	2-pink	-	2
C. krusei	IX	-	-	1	-	-	1
	X	-	-	1	-	-	1
	XI	-	-	1	-	-	1
	XII	-	-	1	-	-	1
C. lusitaniae	IX	-	-	2	1-pink	1-pink	2
	X	-	-	2	2-pink	-	2
	XI	-	-	2	1-pink	1-pink	2
	XII	-	-	2	2-pink	-	2
C. parapsilosis	IX	-	-	1	-	-	1
	X	-	-	1	-	-	1
	XI	-	-	1	-	-	1
	XII	-	-	1	-	-	1
C.tropicalis	IX	2-pink	1-pink		2-mauve	1-pink	-
	X	2-pink	1-pink		2-mauve	1-pink	3

Species	Medium	Coloration at 24 hours: Strong	Weak	None	Coloration at 48 hours: Strong	Weak	None
	XI	2-pink	1-pink		3-pink	-	3
	XII	2-pink	1-pink		3-pink	-	3
S. cerevisiae	IX	-	-	1	-	-	1
	X	-	-	1	-	-	1
	XI	-	-	1	-	-	1
	XII	-	-	1	-	-	1
Trichosporon	IX	-	-	1	1	-	-
	X	-	-	1	-	-	1
	XI	-	-	1	-	-	1
	XII	-	-	1	-	1	-

* : number of strains - color of colonies, "-" = 0

As emerges from Table V above, supplying a combination of a hexosaminidase substrate and a β-glucosidase substrate allows detection of a larger number of yeast species, since it is possible on the media according to the invention to distinguish the *C. albicans* strains, on the one hand, and the *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and *C. tropicalis* strains, on the other hand, from the other yeast species. Media X, XI and XII illustrate the advantage of combining this substrate combination with a hexosaminidase activator, with an inhibitor which is specific for the hexosaminidase of *C. tropicalis* strains or with a mixture of the two. On Medium X, the *C. albicans* strains are detected more quickly than on Medium IX; on Medium XI, the difference between the *C. albicans* strains and the *C. tropicalis* strains is more pronounced and Medium XII combines the advantages of Media X and XI.

CLAIMS

1. Medium for the culturing and specific identification of yeasts, comprising a chromogenic or fluorigenic substrate which can be hydrolyzed by an enzyme of the hexosaminidase family, **characterized in that** the medium also comprises at least one compound which selectively inhibits the hexosaminidase activity of *C. tropicalis*.

2. Medium according to Claim 1, characterized in that the selective inhibitor compound is an amide of formula (I):

(I) $R-(CO-NR'R'')_n$

in which, firstly, either R, R' and R'', independently of each other, consist of:

- a hydrogen atom,
- a saturated or unsaturated, aliphatic or cyclic hydrocarbon-based chain optionally comprising at least one hetero atom,

or each of the radicals R and/or R' and/or R'' together form a cyclic, saturated or unsaturated hydrocarbon-based chain optionally comprising at least one hetero atom,

and, secondly, n is an integer greater than or equal to 1.

3. Medium according to Claim 1, characterized in that the selective inhibitor compound is an amide of formula (I):

(I) $R-(CO-NR'R'')_n$

in which, firstly, either R, R' and R'', independently of each other, consist of:

- a hydrogen atom,
- a saturated or unsaturated, aliphatic or cyclic hydrocarbon-based chain optionally interrupted by at least one hetero atom,

or each of the radicals R and/or R' and/or R'' together form a cyclic, saturated or unsaturated hydrocarbon-based chain optionally interrupted by at least one hetero atom,

and, secondly, n is an integer greater than or equal to 1.

4. Medium according to Claims 1 to 3, characterized in that the selective inhibitor compound is an amide of formula (I):

(I) $R-(CO-NR'R'')_n$

in which, firstly, R, R' and R'', independently of each other, consist of:

- a hydrogen atom,
- an aliphatic hydrocarbon-based chain,

and, secondly, n is equal to 1 or 2.

5. Medium according to Claims 1 to 4, characterized in that the selective inhibitor compound is an acetamide.

6. Medium according to Claims 1 to 5, characterized in that it comprises an activator which is specific for the hexosaminidase enzyme of *C. albicans*.

7. Medium according to Claim 6, characterized in that the activator which is specific for the hexosaminidase enzyme is N-acetylglucosamine.

8. Medium according to the preceding claims, characterized in that it comprises a mixture of selective inhibitor compounds.

9. Medium according to Claim 8, characterized in that the mixture of selective inhibitor compounds consists of acetamide and formamide.

10. Medium according to Claims 1 and 9, characterized in that the medium is gelled and comprises, per liter:

- peptones or a mixture of peptones 0.01-40 g
- yeast extract 0.01-40 g
- glucose (source of carbon) 0-10 g
- phosphate buffer (pH between 5 and 8.5) 2.5-100 mM
- 5-bromo-4-chloro-3-indolyl-N-acetyl-β-D-glucosaminide (Biosynth) $20-600\times10^{-6}$ M
- acetamide 0.01-20 g
- bacterial inhibitor 0-20 g

- agar 11-20 g

11. Medium according to Claims 9 and 10, furthermore comprising N-acetylglucosamine at a concentration of 1.0 g/l.

12. Medium according to Claims 10 and 12, furthermore comprising formamide at a concentration of 0.5 g/l.

13. Medium for the detection and specific identification of yeasts, characterized in that it comprises two substrates, a first chromogenic or fluorigenic substrate which can be hydrolyzed by an enzyme from the hexosaminidase family, and a second chromogenic or fluorigenic substrate which can be hydrolyzed by an enzyme from the glucosidase family.

14. Medium according to Claim 13, in which each substrate consists of a specific portion of the enzyme and of a marker portion, characterized in that the marker portion of the first substrate is different from the marker portion of the second substrate.

15. Medium according to either of Claims 13 and 14, characterized in that it comprises a hexosaminidase activator and/or inhibitor.

16. Medium according to Claim 15, characterized in that the activator consists of a hexosamine and/or a hexosaminidine and/or in that the inhibitor takes the characteristics of any one of Claims 1 to 12.

17. Medium according to any one of Claims 13 to 16, characterized in that the hexosaminidinase substrate consists of an indoxyl derivative and/or in that the glucosidase substrate consists of an indoxyl derivative.

18. Medium according to any one of Claims 1 to 17, characterized in that the medium is liquid or gelled.

19. Microbiological analysis process for selectively identifying the *C. albicans* and/or *C. tropicalis* yeast and/or for differentiating *C. albicans* and *C. tropicalis* yeasts, characterized in that the sample to be analyzed is placed directly in

contact with at least one identification medium according to any one of Claims 1 to 12.

20. Microbiological analysis process for detecting and selectively identifying certain species of *Candida* yeasts, which is characterized in that the sample is placed in direct contact with a medium according to either of Claims 13 and 18, time is allowed for colorations to appear in the medium, and identification is made, on the basis of the differences in coloration, of the *C. albicans* species from, on the one hand, the *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and/or *C. tropicalis* species, and, on the other hand, from the other *Candida* species, and of the *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and/or *C. tropicalis* species from the other *Candida* species.

21. Process according to Claim 20, characterized in that a waiting period of between 36 and 60 hours and advantageously essentially 48 hours is allowed when the medium contains no activator or inhibitor according to either of Claims 15 and 16.

22. Process according to Claim 20, characterized in that a waiting period of between 18 and 30 hours and advantageously essentially 24 hours is allowed when the medium contains an activator or an inhibitor according to either of Claims 15 and 16.

23. Process according to any one of Claims 20 to 22, characterized in that *C. albicans*, *C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and/or *C. tropicalis* are identified from other *Candida* species, when the medium contains:

- a hexosaminidase substrate, and/or
- a glucosidase substrate, and/or
- a hexosaminidase activator, and/or
- a hexosaminidase inhibitor.

24. Process according to any one of Claims 20 to 23, characterized in that *C. albicans* is identified from *C. guilliermondii*, *C. kefyr*, *C. lusitaniae*, *C. tropicalis* and/or other *Candida* species, when the medium contains:

- a hexosaminidase substrate and a glucosidase substrate, and/or
- a hexosaminidase activator, and/or
- a hexosaminidase inhibitor.

DECLARATION AND POWER OF ATTORNEY UNDER 35 USC §371(c)(4) FOR PCT APPLICATION FOR UNITED STATES PATENT

As a below named inventor, I hereby declare that:

my residence, post office address and citizenship are as stated below under my name;

I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought, namely the invention entitled: CULTURE AND IDENTIFICATION ~~MEDIA SPECIFIC~~ OF DIFFERENT SPECIES OF CANDIDA AND ANALYSIS METHODS

described and claimed in international application number PCT/FR98/01717 filed July 31, 1998.

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations §1.56. Under Title 35, U.S. Code §119, the priority benefits of the following foreign application(s) filed within one year prior to my international application are hereby claimed:

FR 97.10635 filed on August 20, 1997

FR 98.05269 filed on April 20, 1998

The following application(s) for patent or inventor's certificate on this invention were filed in countries foreign to the United States of America either (a) more than one year prior to my international application, or (b) before the filing date of the above-named foreign priority application(s):

I hereby appoint the following as my attorneys of record with full power of substitution and revocation to prosecute this application and to transact all business in the Patent Office:

James A. Oliff, Reg. No. 27,075; William P. Berridge, Reg. No. 30,024; Kirk M. Hudson, Reg. No. 27,562; Thomas J. Pardini, Reg. No. 30,411; and Edward P. Walker, Reg. No. 31,450.

ALL CORRESPONDENCE IN CONNECTION WITH THIS APPLICATION SHOULD BE SENT TO OLIFF & BERRIDGE, P.O. BOX 19928, ALEXANDRIA, VIRGINIA 22320, TELEPHONE (703) 836-6400.

I hereby declare that I have reviewed and understand the contents of this Declaration, and that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1 Typewritten Full Name of Sole or First Inventor: ORENGA (Family Name) Sylvain (Given Name) (Middle Initial)

2 Inventor's Signature: Orenga Sylvain

3 Date of Signature: 9 Feb 2000

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Note to Inventor: Please sign name on line 2 exactly as it appears in line 1 and insert the actual date of signing on line 3.

IF THERE IS MORE THAN ONE INVENTOR USE PAGE 2 AND PLACE AN "X" HERE ☐

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